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14. ABSTRACT
We proposed to define the quantitative relationship between inherited risk of developing mammary cancer and the number of MaSC under basal and hormone stimulated states in two well characterized inbred rat strains: 1) ACI, which is highly susceptible to 17β-estradiol (E2)-induced/progesterone (P)-dependent mammary cancer; and 2) Brown Norway (BN), which is highly resistant. Major findings: ACI and BN rats likely exhibit significant differences in MaSC number and/or responsiveness to hormone. Technical issues and loss of commercial source of BN rats led to revision of Aims. The data generated indicate that the susceptible ACI and resistant BN rat strains exhibit fundamental differences in their cellular response programs to E2. Whereas the mammary epithelium of the ACI proliferates in response to E2, the mammary gland of BN rats shows a slight proliferative response coupled with differentiation to secretory epithelium. We believe this difference in the cellular response to E2 stems from differences in MaSC and/or MaPC numbers or responsiveness to hormones, and that this these differences in turn explain, at least in part, the differing susceptibilities of these rat strains to E2-induced mammary cancer.

15. SUBJECT TERMS
Breast cancer, estrogen-induced susceptibility, rat models

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INTRODUCTION

We proposed to test the hypothesis that inherited risk of breast cancer is determined, at least in part, by genetically determined quantitative differences in the ability of E2 and P to regulate mammary stem cell (MaSC) and/or mammary progenitor cell (MaPC) number and/or function. Our approach was to develop methods for quantifying MaSC in two well characterized rat strains, ACI and BN, which differ dramatically from one another in terms of their susceptibility to 17β-estradiol (E2)-induced mammary cancer. Aim 1 was to quantify MaSC numbers in ACI and BN rats by performing limiting dilution transplantation assays into the cleared mammary fad pads of (ACIxBN)F1 recipient rats. Aim 2 was to quantify MaSC and MaPC in ACI and BN rats using established *in vitro* colony forming assays.

BODY

Development of methods.

The first step of this research was to develop procedures for isolating and characterizing defined populations of cells from the rat mammary gland. Using published work on the identification and characterization of defined cell populations in the mouse mammary gland as our guide, we established flow cytometry based methods for sorting rat mammary epithelial cells into four distinct populations based on expression of cell surface markers CD45 (BD Biosciences, Ab #554878), CD31 (BD Biosciences, Ab #555027), CD24 (BD Biosciences, Ab # 551133) and CD29 (AbD Serotech, Ab # MCA2298FT). Procedures were then established for transplanting isolated mammary epithelial cells into the interscapular fat pad. The number of mammary gland outgrowths expressed as a function of the number of cell transplanted provides the basis for quantification of MaSC number. Finally, methods were established for primary culture of mammary epithelial cells to allow the number of colony forming units, an indicator of MaPC number, to be quantified.

Results from original objectives.

Identification of strain differences in mammary epithelial cell populations. Employing methods developed above, we quantified the relative numbers of defined mammary epithelial cell types between susceptible ACI and resistant BN rats (Figure 1). Data from these experiments indicate that the numbers of putative luminal ($CD29^{low}CD24^{+}$) and putative basal ($CD29^{high}CD24^{+}$) epithelial cells, relative to other mammary cell types, are higher in susceptible ACI rats than in resistant BN rats.

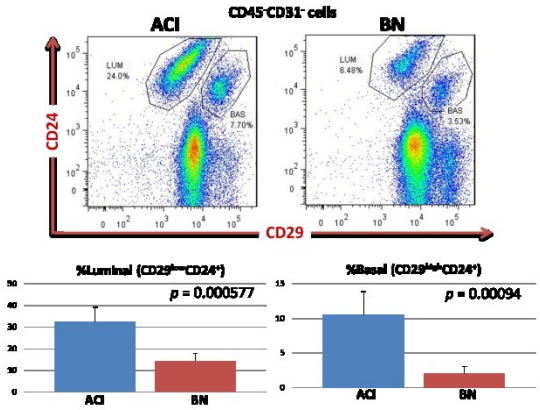
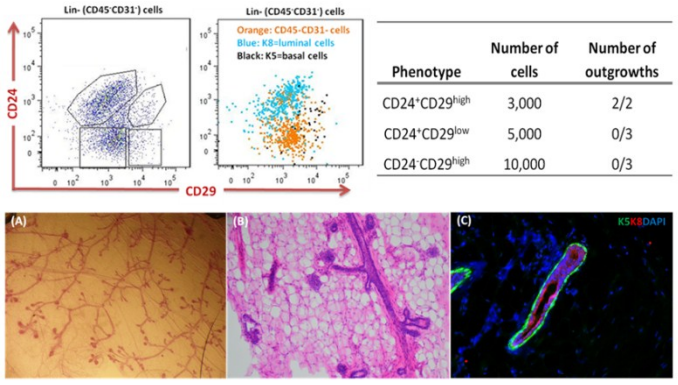


Figure 1. Quantification of Luminal and Basal Epithelial Cells. Representative flow cytometry data are presented to illustrate the relative numbers of luminal ($CD29^{low}CD24^{+}$) and basal ($CD29^{high}CD24^{+}$) epithelial cells in the mammary glands of untreated, ovary intact, ACI and BN rats. The bar graphs present data from three independent experiments and illustrate that the relative numbers of luminal and basal epithelial cells are higher in ACI rats, compared to BN rats.

Identification of MaSC in basal epithelial cell population. We demonstrated that the $CD29^{low}CD24^{+}$ cells express cytokeratin 8, a marker of luminal epithelial cells, whereas the $CD29^{high}CD24^{+}$ cells express cytokeratin 5, a marker of basal epithelial cells (Figure 2, upper left). These data confirm the preliminary identification of these cell populations as described in Figure 1. Moreover, only the $CD29^{high}CD24^{+}$ basal epithelial cells were capable of generating mammary outgrowths when transplanted into the interscapular fat pad of recipient rats (Figure 2, upper right). These mammary outgrowths were comprised of elongated ducts with terminal and lateral buds (Figure 2, lower panels A (whole mount) and B (hematoxylin/eosin stained thin section). The mammary outgrowths were well organized at the cellular level, and consisted of cytokeratin 8 positive luminal epithelial cells (red), surrounded by cytokeratin 5 positive basal epithelial cells (green) (Figure 2, lower panel C). Blue staining (DAPI) represents DNA in cell nuclei.



Phenotype	Number of cells	Number of outgrowths
$CD24^{+}CD29^{high}$	3,000	2/2
$CD24^{+}CD29^{low}$	5,000	0/3
$CD24^{-}CD29^{high}$	10,000	0/3

Figure 2. Identification of Cell Population Harboring Mammary Stem Cells. Mammary cells were sorted into defined populations based on expression of CD45, CD31, CD24 and CD29. The $CD29^{low}CD24^{+}$ cells express cytokeratin 8, a marker of luminal epithelial cells, whereas the $CD29^{high}CD24^{+}$ cells express cytokeratin 5, a marker of basal epithelial cells. Mammary gland outgrowths were generated upon transplantation of the $CD29^{high}CD24^{+}$ cell population, indicating MaSC reside within this population. The outgrowths generated upon transplantation closely resemble normal mammary gland in their cellular organization.

Identification of genetic polymorphism in CD45. To further validate our flow cytometry based methods we evaluated the nucleotide sequences of the ACI and BN alleles of the genes encoding the cell surface markers used in our sorting protocol, recognizing that polymorphisms that impact the amino acid sequences of these cell surface markers could potentially alter antibody binding affinities and thereby compromise the ability of these antibodies to be used in quantitative assays of specific cell populations. A coding region polymorphism was identified in the gene encoding CD45 (official gene symbol and names are *Ptprc* and *Protein Tyrosine Phosphatase Receptor Type C*). A C in the second position of codon 74 results in incorporation of a histidine residue in place of an arginine residue in the CD45/Ptprc protein. Although this conservative amino acid substitution is unlikely to impact CD45/Ptprc protein function, it could possibly alter antibody binding affinity to the cell surface protein. The supplier of the antibody to CD45 will not disclose the epitope used in the generation of the antibody. Therefore, we must determine experimentally whether or not the observed polymorphism impacts binding of the antibody to the ACI and BN variants of the CD45/Ptprc protein. Once this observation was made, we placed a hold on the use of flow based protocols until this important control work can be been completed.

Unanticipated pitfall: loss of commercial source of BN rat strain in United States.

During the performance of this project, Harlan, the only supplier of BN rats in the U.S., discontinued selling these animals and eliminated their breeding colony in the U.S. Because our research is dependent upon BN rats, we had to establish a breeding colony in our laboratory to produce these animals. Our ability to produce the needed animals is limited due to time needed to establish breeding stock and produce offspring, and also by space and financial constraints. Therefore, it was necessary to revise our objectives to complete experiments that are directly related to our original hypothesis but that require fewer BN rats.

Results from revised objectives.

ACI and BN rats exhibit markedly difference early responses to estrogen.

The preliminary data summarized above suggest that the relative numbers of mammary cell types differ between susceptible ACI and resistant BN rats. Based on these data, we hypothesized that the cellular responses in the mammary gland would differ between these strains at early time points following initiation of treatment with E2.

Dramatic differences in mammary gland cellular responsiveness to E2 was observed within 1 week and remained apparent following 3 and 12 weeks of E2 treatment. Figure 3 illustrates mammary gland whole mounts from ACI and BN rats, both sham treated control and E2 treated. Figure 4 illustrates hematoxylin and eosin (H&E) stained thin sections. A robust epithelial hyperplasia was induced by E2 in ACI rats at all three time points. By contrast, E2 induced a modest hyperplasia and ductal ectasia in the BN rat strain.

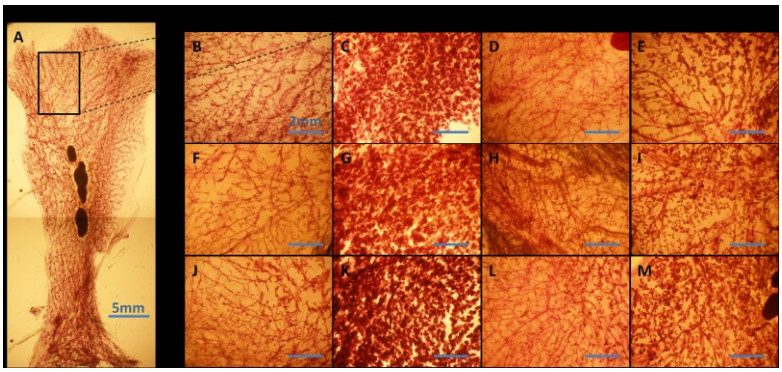


Figure 3. Mammary gland whole mounts prepared from sham treated control and E2 treated ACI and BN rats. E2 induced dramatic epithelial hyperplasia in ACI rats. By contrast, E2 induced a modest epithelial hyperplasia in BN rats.

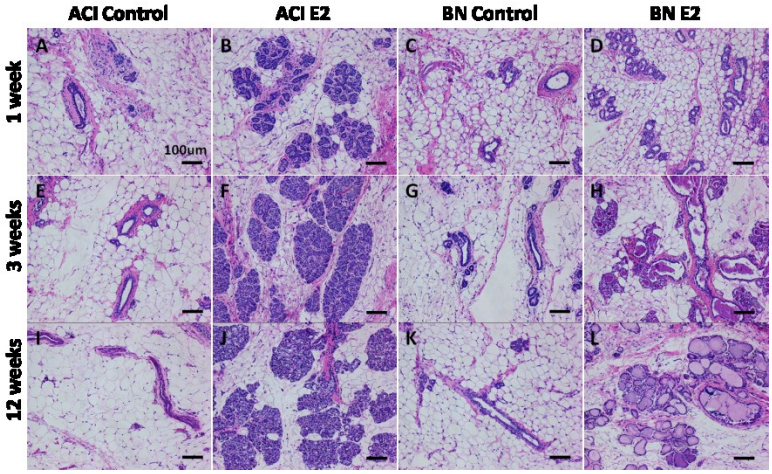


Figure 4. H&E stained sections of mammary gland prepared from sham treated control and E2 treated ACI and BN rats. E2 induced dramatic epithelial hyperplasia in ACI rats. By contrast, E2 induced a modest epithelial hyperplasia and ductal ectasia in BN rats.

The strain specific responses to E2 were evaluated further to define the responding mammary cell populations. Using immunohistochemistry (IHC)-based approaches, we demonstrated that E2 induces proliferation in the luminal epithelial cell compartment as evidenced by co-staining with antibodies to cytokeratin 8 and BrdU, a thymidine analog that was injected 4 hours prior to euthanasia to label cells in S phase of the cell cycle (Figure 5). The IHC data were quantified using a Vectra Multispectra Image Analysis System. These quantitative analyses indicated that E2 stimulated luminal epithelial proliferation 6 to 8-fold in ACI rats, but only approximately 2-fold in BN rats (Figures 5 and 6).

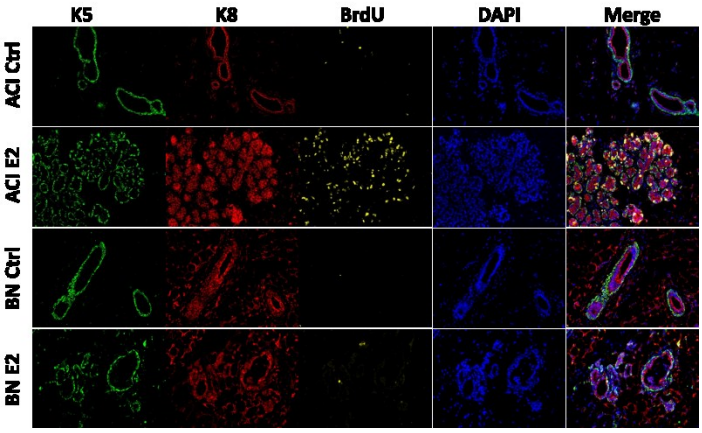


Figure 5. E2 markedly stimulates luminal epithelial cell proliferation in ACI rats, but not BN rats. Mammary tissues were harvested three weeks following initiation of E2 treatment. Basal epithelial cells were identified by expression of cytokeratin 5 (K5), luminal epithelial cells by expression of cytokeratin 8 (K8), S phase cells by incorporation of BrdU, and nuclei by staining of DNA using DAPI. Merged images reveal a high fraction of S phase luminal epithelial cells in E2 treated ACI, but not BN, rats.

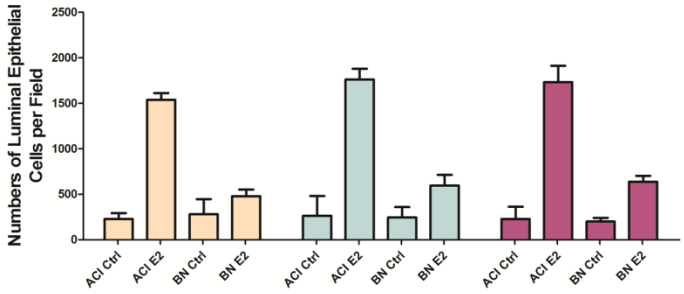


Figure 6. Quantitative image analyses reveal marked strain difference in response to estrogen. E2 induced a 6 to 8-fold increase in mammary luminal cell proliferation in ACI rats, but only an approximate 2-fold increase in BN rats.

We next performed IHC using antibodies generated against a cocktail of milk proteins. The mammary glands of untreated and E2 treated ACI rats exhibited only modest immunostaining in the ductal lumina. By contrast, the glands of the E2 treated BN rats exhibited marked immunostaining to milk proteins (Figure 7). Together, the data presented in this section strongly suggest that the susceptible ACI and resist BN rat strains exhibit fundamental differences in their response programs to E2. Whereas the mammary epithelium of the ACI proliferates in response to E2, the mammary gland of BN rats shows a slight proliferative response coupled with differentiation to secretory epithelium. We believe this difference in the cellular response to E2 stems from differences in MaSC and/or MaPC numbers or responsiveness to hormone, and that this these differences in turn explain, at least in part, the differing susceptibilities of these rat strains to E2-induced mammary cancer. These associations will be tested mechanistically in future studies.

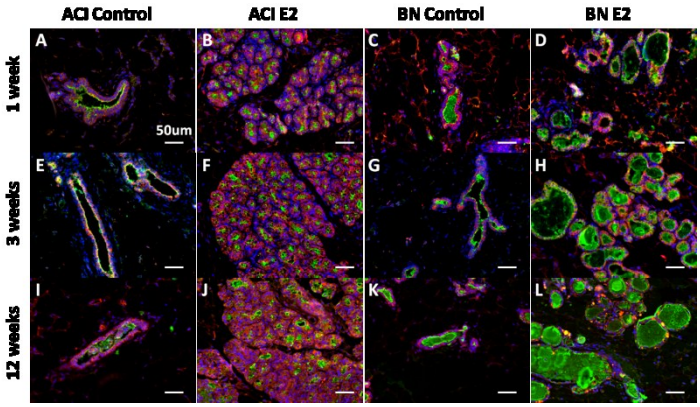


Figure 7. Quantitative image analyses reveal marked strain difference in response to estrogen. E2 induced a 6 to 8-fold increase in mammary luminal cell proliferation in ACI rats, but only an approximate 2-fold increase in BN rats.

KEY RESEARCH ACCOMPLISHMENTS

1. Methods were developed for using flow cytometry and FACS to identify, isolate and characterize specific mammary cell populations.

2. Rat luminal epithelial cells (like mouse) exhibit a $CD31^{-}CD45^{-}CD24^{+}CD29^{low}$ phenotype.

3. Rat basal epithelial cells (like mouse) exhibit a $CD31^{-}CD45^{-}CD24^{+}CD29^{high}$ phenotype.

4. ACI and BN rats appear to exhibit relative differences in luminal and basal epithelial cell numbers.

4. The $CD31^{-}CD45^{-}CD24^{+}CD29^{high}$ basal epithelial cell population is enriched for MaSC.

5. A coding region polymorphism in CD45/Ptprc resulting from a nucleotide variant between the ACI and BN rat strains was identified. This finding necessitated the development and performance of additional control experiments to validate the chosen experimental approach and a revision of aims, objectives and timeline.

REPORTABLE OUTCOMES

Manuscripts: one in preparation

Abstracts: one

> Ding, L., Hickman, M., Seiler, N, Colletti, J., Warren, C., Ozers, M., Becker, N., Shull, J.D. Genetic and Cellular Bases of Susceptibility to Estrogen-Induced Mammary Cancer. Cold Spring Harbor Laboratory Meeting, Rat Genomics & Models, Dec 7-10, 2011. (Shull, invited oral presentation)

Presentations: one

> Genetic and Cellular Bases of Susceptibility to Estrogen-Induced Mammary Cancer. University of Louisville, Department of Biochemistry and Molecular Biology, Feb 27, 2012. (Shull, invited oral presentation)

Patents/licenses: none

Degrees supported: one doctoral student support, training in progress

Cell lines, tissue repositories, animal models, *etc*.: none

Funding applied for: an R01 application to the NIH is in development

Employment and research opportunities: none

CONCLUSION

The data generated in association with this award strongly suggest that the susceptible ACI and resist BN rat strains exhibit fundamental differences in their cellular response programs to E2. Whereas the mammary epithelium of the ACI proliferates in response to E2, the mammary gland of BN rats shows a slight proliferative response coupled with differentiation to secretory epithelium. We believe this difference in the cellular response to E2 stems from differences in MaSC and/or MaPC numbers or responsiveness to hormone, and that this these differences in turn explain, at least in part, the differing susceptibilities of these rat strains to E2-induced mammary cancer.

REFERENCES

None

APPENDICES

None